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Involvement of connective tissue growth factor (CTGF) in insulin-like growth factor-I (IGF1) stimulation of proliferation of a bovine mammary epithelial cell line

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Abstract

The objective of this study was to determine the mechanism by which insulin-like growth factor-I (IGF1) stimulates proliferation of mammary epithelial cells, using the bovine mammary epithelial cell line MAC-T as a model. IGF1 significantly up- or down-regulated the expression of 155 genes in MAC-T cells. Among the most significantly suppressed was the gene for connective tissue growth factor (CTGF), a secretory protein that has both proliferative and apoptotic effects and is also a low-affinity binding protein of IGF1. IGF1 inhibited CTGF expression through the PI3K-Akt signaling pathway. Administration of growth hormone (GH), a strong stimulator of IGF1 production in vivo, decreased mammary CTGF mRNA in cattle; however, GH did not affect CTGF expression in MAC-T cells, suggesting that IGF1 may also inhibit CTGF expression in the mammary gland. Added alone CTGF stimulated proliferation of MAC-T cells, but in combination with IGF1 it attenuated IGF1's stimulation of proliferation of MAC-T cells. Excess IGF1 reversed this attenuating effect of CTGF. Despite being an IGF binding protein, CTGF did not affect IGF1-induced phosphorylation of IGF1 receptor (IGF1R) or IGF1R expression in MAC-T cells, indicating that the attenuating effect of CTGF on IGF1 stimulated proliferation of MAC-T cells was not mediated by decreasing IGF1's ability to bind to IGF1R or by decreasing IGF1R expression. Overall, these results suggest a novel biochemical and functional relationship between CTGF and IGF1 in the bovine mammary gland, where IGF1 may inhibit CTGF expression to reduce the attenuating effect of CTGF on IGF1 stimulated proliferation of epithelial cells.

Keywords: IGF1; Cell proliferation; Mammary; Bovine; CTGF

1. Introduction

Insulin-like growth factor-I (IGF1) is a growth factor that stimulates growth and proliferation and inhibits apoptosis of a variety of cell types, including fibroblast, muscle, epithelial, and endothelial cells [1]. The actions of IGF1 start with binding to the IGF1 receptor (IGF1R), a member of the tyrosine kinase receptor family [1,2]. Binding of IGF1 to IGF1R activates the receptor kinase, and the receptor phosphorylates itself and two major substrates, insulin receptor substrate (IRS) and Src homologous and collagen protein (SHC) [2]. The phosphorylated IRS and SHC then activate two signaling pathways, the phosphatidylinositol (PI) 3-kinase pathway and the MAP kinase pathway, respectively. These two pathways, each involving multiple

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intracellular protein or lipid molecules, lead to changes in gene transcription, protein synthesis, and protein activity that are ultimately responsible for the effects of IGF1 on cell proliferation, differentiation, apoptosis, and metabolism [2].

IGF1 is produced by a wide spectrum of tissues and cells, and is present in the blood at high concentrations [1]. The circulating IGF1 is mainly secreted by the liver under the control of growth hormone (GH), and is believed to mediate at least some of the effects of GH on growth and development [3,4]. The availability of IGF1 to IGF1R is modulated by many factors, including six IGF binding proteins, IGFBP1–IGFBP6 [5], which bind IGF1 with high affinities, and at least nine IGFBP-related proteins (IGFBP-rPs), which are structurally similar to IGFBPs but bind IGF1 at lower affinities [6]. Most of the IGFBP-rPs were initially not identified as IGF binding proteins and hence had unique names. For example, IGFBP-rP2 was identified as connective tissue growth factor (CTGF) produced by human vascular endothelial cells [7], and was later found to contain a motif homologous to IGFBPs and to specifically bind IGF1 [8]. However, unlike the high-affinity IGFBPs, the physiological roles of IGFBP-rPs in the IGF system are largely unknown [6]. IGF1 plays an important role in mammary gland development and lactation. Deletion of the IGF1 gene prevents normal terminal bud formation and ductal morphogenesis [9], whereas over expression of IGF1 stimulates alveolar bud development in the mammary gland of transgenic mice [10]. Many studies also suggest that IGF1, produced locally or secreted from the liver, mediates the effect of GH on mammary gland development and milk production in cows [11]. These effects of IGF1 appear to be mediated at least in part through stimulation of proliferation of the mammary epithelial cells [11].

This study was conducted as one of the steps toward understanding the mechanism by which IGF1 stimulates mammary gland development in cows. In this study, we used the bovine mammary epithelial cell line MAC-T cells as a model for the bovine mammary epithelial cells, as the MAC-T cells retain many of the characteristics of mammary epithelial cells in vivo [12] and are responsive to IGF1 [13,14]. We started this study by identifying genes that are regulated by IGF1 in MAC-T cells. Among the genes that were markedly down-regulated by IGF1 was CTGF. Connective tissue growth factor (also called CCN2) is a member of the recently characterized CCN family [15]. The members of this family are cysteine-rich and contain four conserved domains: an IGF binding domain, a von Willebrand type C domain, a thrombospondin-1 domain, and a putative cystine knot-containing C-terminal domain, and are secreted proteins [15]. Because CTGF is both an IGF binding protein named IGFBP-rP2 [8] and a growth factor [15], we conducted additional experiments to evaluate a potential role of CTGF in IGF1 stimulation of proliferation of MAC-T cells, and the signaling pathway that mediates IGF1 inhibition of CTGF expression in those cells.

2. Materials and methods

2.1. Cell culture and treatments

The MAC-T cells, originally obtained from Dr. J.D. Turner [12], were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM of L-glutamine, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% of fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO_2. The medium, FBS and antibiotics were purchased from Sigma–Aldrich (St. Louis, MO).

To determine the effect of IGF1 on mRNA expression in MAC-T cells, the cells were seeded in 100-mm dishes and cultured for 24 h. The cells were then cultured in serum-free medium (10 mL) for 8 h and subsequently cultured under one of the following treatments for 16 h: (1) 200 ng/mL of recombinant human IGF1 (Sigma–Aldrich), which is identical in amino acid sequence to bovine IGF1; (2) 50 μM of LY294002 (Calbiochem, San Diego, CA), a PI3-kinase inhibitor; (3) 50 μM of PD098059 (Calbiochem), a MEK inhibitor; (4) 20 nM of rapamycin (Sigma–Aldrich), a p70 S6 kinase inhibitor; (5) 200 ng/mL of IGF1 and 20 nM of rapamycin; (6) 200 ng/mL of IGF1 and 50 μM of LY294002; (7) 200 ng/mL of IGF1 and 50 μM of PD098059; and (8) 50 μL of phosphate buffered saline (PBS) and 50 μL of dimethyl sulfoxide (DMSO), the vehicles for IGF1 and signaling inhibitors, respectively. The concentration of IGF1 used in this study was within the range of its physiological concentrations in cattle [16]. At the described concentrations, LY294002, PD098059, and rapamycin were expected to completely block the signaling pathways from IGF1R [17–19].

To determine the effect of IGF1 or CTGF on proliferation of MAC-T cells, the cells grown in 100-mm dishes at ∼60% confluency were split into 96-well plates at 1×10^4 cells/well; the cells were cultured in medium containing 10% FBS for 4 h followed by serum starvation for 8 h. The cells were subsequently treated with 200 ng/mL of IGF1, 200 mg/mL of recombinant human CTGF (Fitzgerald, Concord, MA), which is 93% identical in amino acid sequence to bovine CTGF,

combinations of increasing concentrations of IGF1 and 200 mg/mL of CTGF, or 10% FBS (serving as a positive control) for 16 h, followed by cell proliferation assay.

To determine the effect of IGF1 and CTGF, alone or combination, on phosphorylation of IGF1R, MAC-T cells were treated with 200 ng/mL of IGF1, 200 ng/mL of CTGF, or 200 ng/mL of IGF1 and 200 ng/mL of CTGF for 1 h before being lysed for Western blotting analysis.

2.2. Cell proliferation assay

The cell proliferation assay was performed using the Nonradioactive CellTiter 96 Assay kit (Promega, Madison, WI), essentially according to the manufacturer's instructions. Briefly, the cells in each well were treated with 15 μL of dye solution from the kit for 4 h. Then 100 μL of solubilization/stop solution (from the kit) was added and the plate was incubated overnight before the absorbance at 570 nm was recorded using a 96-well plate reader. This cell proliferation experiment was repeated four times, each time in four replicates.

2.3. Microarray analysis

Total RNA from MAC-T cells treated with 200 ng/mL of IGF1 or PBS (control) for 16 h was isolated using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH). The RNA was further purified using the RNeasy MiniElute Cleanup kit (Qiagen, Valencia, CA). RNA quality was confirmed using the Agilent Bioanalyzer 2100. Four IGF1- and four PBS-treated MAC-T RNA samples were used in a microarray analysis. The microarray analysis (RNA labeling, hybridization, and scanning) was conducted by the Virginia Tech Core Laboratory Facility, using the Affymetrix Genechip Bovine Genome Arrays that contained ~23,000 bovine transcripts (Affymetrix, West Sacramento, CA). The microarray data were analyzed using the Genesifter software (http://www.genesifter.net/web/) from VizX Labs (LLC, Seattle, WA). The data were normalized on global mean and $\log_2$-transformed. The transformed data were analyzed for statistical significance using *t*-test with Benjamini and Hochberg adjustment [20]. A false discovery rate of 5% was used as a cutoff for statistical significance. An expression difference was considered significant if it was at least 2-fold and the associated *P* value was less than 0.05.

2.4. Quantitative real-time RT-PCR

Two μg of total RNA were reverse-transcribed in a total volume of 10 μl using TaqMan Reverse Transcribing Reagents (Applied Biosystems, Foster City, CA), for 10 min at 25 °C, 30 min at 48 °C, and 5 min at 95 °C. Two μL of the reverse-transcribed products were amplified in a total volume of 25 μl containing 12.5 μL of SyberGreen PCR Master Mix (Applied Biosystems) and 0.2 μM of gene-specific forward and reverse primers (Table 1) under 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The real-time PCR data were analyzed using the $2^{-\Delta\Delta C_t}$ method [21], using GAPDH mRNA as an internal control. Based on the C_t values, the expression of GAPDH mRNA was not different across the treatments in this study.

2.5. Western blotting analysis

The MAC-T cells were treated with IGF1 and CTGF, alone or in combination, as described above. The cellular protein lysates were prepared by lysing the cells in a buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% NP-40, and protease inhibitors (Roche, Indianapolis, IN). Total protein concentrations were determined using the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA), according to the manufacturer's instructions. 40 μg of cellular protein lysates were separated by electrophoresis in a 12% SDS–polyacrylamide gel and then transferred to a nitrocellulose membrane (Bio-Rad). After blocking with 5% nonfat dried milk in TBST that contained 20 mM Tris–HCl (pH 7.5), 500 mM NaCl, and 0.05% Tween 20 for 3 h, the membrane was incubated with 1:1000 diluted rabbit anti-phospho-IGF1R antibody (Cell Signaling, Boston, MA) at 4 °C overnight. After being washed three times in TBST, the membrane was incubated with 1:2000 diluted horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at room temperature. The membrane was subsequently incubated in SuperSignal West Pico Chemiluminescence Substrate (Pierce Biotechnology, Rockford, IL) for 5 min, and the chemiluminescent signals were detected by exposure to X-ray films. The membrane was then stripped by immediately incubating the membrane in Restore Western Blot Stripping Buffer (Pierce Biotechnology, Rockford, IL) for 30 min at room temperature. After blocking and washing, the membrane was incubated with 1:1000 diluted rabbit anti-IGF1R antibody (Cell Signaling) at 4 °C overnight. The same membrane was stripped and re-probed with an anti-beta-actin antibody (Cell Signaling). The intensities of phospho-IGF1R, total IGF1R, and beta-actin bands were measured using the ImageJ program (http://rsb.info.nih.gov/ij/). The band intensity of phospho-IGF1R protein or total

Table 1
Primers used in real-time PCR quantification of mRNA abundance

Gene symbol	Gene description	GenBank accession #	Sequences of primers[a]	Product size (bp)
CTGF	Connective tissue growth factor	BC113279.1	AGCTGACCTGGAGGAGAACA GTCTGTGCACACTCCGCAGA	139
CCND1	Cyclin D1	NM001046273.1	GCACTTCCTCTCCAAGATGC GTCAGGCGGTGATAGGAGAG	204
CCND2	Cyclin D2	NM001076372.1	CCAGACCTTCATCGCTCTGT GATCTTTGCCAGGAGATCCA	163
SLC1A5	Solute carrier family 1, member 5	BC123803.1	TCGATTCGTTCCTGGATCTT CCAGGCCCAGAATGTTCATA	162
FABP3	Fatty acid binding protein 3	BT021486.1	TGCAGAAGTGGAATGGACAA GCAGTCAGTGGAAGGAGAGG	145
DDIT4	DNA-damage-inducible transcript 4	NM001075922.1	ACAGCAGCAACAGTGGCTTT ACCTGGCTCACCAGCTGAC	208
IGFBP3	Insulin-like binding protein 3	BC149336	CAGAGCACAGACACCCAGAA TGCCCCGTACTTATCCACACA	231
BCL2	B-cell leukemia/lymphoma 2	XM586976.3	CCTGTGGATGACCGAGTACC CCTTCAGAGACAGCCAGGAG	134
CSN2	Casein beta	NM_181008	GTGAGGAACAGCAGCAAACA TTTTGTGGGAGGCTGTTAGG	115
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	XM001252479	GGGTCATCATCTCTGCACCT GGTCATAAGTCCCTCCACGA	177

[a] All sequences are written from 5′ to 3′. The upper sequence of each pair of sequences is for the forward primer and the lower for the reverse primer.

IGF1R protein in a sample was divided by that of beta-actin to normalize potential variation in protein loading.

2.6. Mammary tissue

Mammary parenchymal tissues from 12 nonpregnant lactating Holstein cows taken in a previous study [22] were used in this study. Six cows were injected intramuscularly with 40 mg/day of recombinant bovine GH (sometribove, provided by Monsanto Company, St. Louis, MO) for 5 consecutive days, and the other 6 cows with an equal volume (4 mL) of excipient. The mammary tissue was collected at slaughter, 20–23 h after the last injection. Total RNA from the tissue samples was isolated using TRI reagent. The quality of the RNA was confirmed by gel electrophoresis.

2.7. Cell transfection

To determine the effect of GH on CTGF mRNA expression in MAC-T cells, the cells in 6-well plates were transfected with 2 μg of a GHR expression plasmid and 2 μg of a STAT5b expression plasmid using FuGene 6 (Roche) as described previously [23]. These transfections made the MAC-T cells highly responsive to GH [23]. Twenty-four hours after the transfection, the cells were serum-starved for 8 h, and subsequently treated with 500 ng/mL of recombinant bovine GH (provided by Dr. A.F. Parlow, National Hormone and Peptide Program, Torrance, CA) or PBS, for 16 h. Total RNA was isolated from the cells, and CTGF and GAPDH mRNA expression was quantified by quantitative real-time RT-PCR.

2.8. Statistical analysis

The statistical analysis of the microarray data was described above. The cell proliferation data were analyzed using ANOVA followed by the Tukey test, which were performed using the General Linear Model of SAS (SAS Inst., Inc., Cary, NC). The mRNA expression data from quantitative real-time RT-PCR or protein expression data from Western blotting analysis were analyzed by *t*-test. A difference was considered statistically significant when $P < 0.05$, and not significant when $P > 0.1$. All

Table 2
Top 10 *IGF1* up- or down-regulated genes in MAC-T cells

Probe ID[a]	GenBank #[b]	Ratio[c]	Gene symbol	Gene description
Up-regulated genes				
bt.2091.1.s1_at	CK846550	4.64	DDIT4	DNA-damage-inducible transcript 4
bt.22763.1.s1_at	CK974002	4.58	HMGCS1	3-Hydroxy-3-methylglutaryl-coenzyme A synthase 1
bt.4758.1.s1_at	NM_174313	3.91	FABP3	Fatty acid binding protein 3
bt.13588.3.a1_at	CF763176	3.25	MGC166278	Phosphoserine aminotransferase 1
bt.6465.1.s1_at	CK960423	3.18	MGC128356	Cytochrome *c*, somatic
bt.4733.1.s1_at	CK771294	3.07	PHGDH	Phosphoglycerate dehydrogenase
bt.9767.1.s1_a_at	CK949309	2.97	SQLE	Squalene monooxygenase
bt.9957.1.s1_a_at	CK847195	2.93	MGC143283	14-3-3 protein sigma (Stratifin)
bt.2073.1.s1_at	CK972168	2.87	LOC533333	Hypothetical protein
bt.9078.2.s1_a_at	BI898927	2.79	LOC505916	Spermidine synthase
Down-regulated genes				
bt.16875.1.a1_at	CB424375	0.17	LOC541012	T-complex 11 like 2
bt.5240.1.s1_at	NM_174030	0.22	CTGF	Connective tissue growth factor
bt.11993.1.s1_at	CK953351	0.22	MGC137132	hydroxysteroid (17-beta) dehydrogenase 11
bt.15836.1.s1_at	CB168998	0.22	ANKRD1	Ankyrin repeat domain 1 (cardiac muscle)
bt.12327.1.s1_at	CK849102	0.25	MGC154995	Thioredoxin interacting protein
bt.24310.1.s1_at	BP103230	0.27	Unknown	Transcribed locus
bt.20501.1.s1_at	BF774834	0.29	Unknown	Transcribed locus
bt.24316.1.a1_at	BP106018	0.30	FBXO32	F-box protein 32
bt.27487.1.a1_at	CK846214	0.31	Unknown	Transcribed locus
bt.3211.1.s1_at	CB440509	0.31	GABARAPL1	GABA(A) receptor-associated protein like 1

[a] Affymetrix probe ID.
[b] GenBank accession number on which Affymetrix probe was based.
[c] Ratio of mRNA abundance treated with *IGF1* to that treated with PBS.

data were expressed as mean ± S.E.M. (standard error of the mean).

3. Results

3.1. Effects of IGF1 on mRNA expression in MAC-T cells

To identify genes that are regulated by IGF1 in MAC-T cells, mRNA profiles in IGF1- and PBS-treated MAC-T cells were compared by a microarray analysis. 155 transcripts, or 0.67% of the 23,000 bovine transcripts probed, were differentially (≥2-fold, $P < 0.05$) expressed between the two treatments, with 81 transcripts being up-regulated and 74 transcripts down-regulated by IGF1. Examples of the genes most significantly up-regulated by IGF1 were DNA-damage-inducible transcript 4 (DDIT4), 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1 (HMGCS1), and fatty acid binding protein 3 (FABP3) (Table 2). Examples of the genes most significantly down-regulated by IGF1 were T-complex 11 like 2 (LOC541012), connective tissue growth factor (CTGF), and hydroxysteroid (17-beta) dehydrogenase 11 (MGC137132) (Table 2).

To validate the microarray data, the expression of nine mRNAs was measured by quantitative real-time RT-PCR (Table 3). These nine mRNAs were selected based on their potential involvement in mediating (e.g., CCND1, CCND2, DDIT4, BCL2, SLC1A5, and FABP3) or modulating (e.g., CTGF, IGFBP3) the effects of IGF1 on cell proliferation or cell metabolism, or as an indicator of secretory activity (CSN2). The PCR data were in general consistent with the microarray data for these mRNAs (Table 3). For example, both PCR and microarray analyses indicated that IGF1 reduced CTGF, increased FABP3, DDIT4, CCND2, and IGFBP3, and had no significant effects on expression of BCL2 and CCND1 mRNAs in MAC-T cells (Table 3). The observation that IGF1 stimulated IGFBP3 mRNA expression in MAC-T cells is also consistent with a previous study [24].

3.2. Signaling pathway mediating IGF1 inhibition of CTGF expression

Because CTGF was recently characterized as an IGF binding protein [8], we further identified the signaling pathway by which IGF1 inhibited CTGF mRNA expression in MAC-T cells. As shown in Fig. 1, PD98059, a

Table 3
Validation of microarray data by real-time PCR of nine mRNAs

Gene symbol	Gene description	Ratio of mRNA abundance[a]	
		By microarray (*P* values)	By real-time PCR (*P* values)
CTGF	Connective tissue growth factor	0.22 (<0.05)	0.15 (<0.01)
CCND1	Cyclin D1	1.50 (>0.05)	0.77 (>0.05)
CCND2	Cyclin D2	2.25 (=0.05)	1.59 (<0.05)
SLC1A5	Solute carrier family 1 (neutral amino acid transporter), member 5	1.73 (>0.05)	1.20 (>0.05)
FABP3	Fatty acid binding protein 3	3.91 (<0.05)	4.07 (<0.05)
DDIT4	DNA-damage-inducible transcript 4	4.64 (<0.05)	9.21 (<0.05)
IGFBP3	Insulin-like growth factor binding protein 3	1.94 (<0.05)	6.40 (<0.01)
BCL2	B-cell leukemia/lymphoma 2	1.32 (>0.05)	0.99 (>0.05)
CSN2	Casein beta	Undetectable	0.99 (>0.05)

[a] Ratio of mRNA abundance treated with *IGF1* to that treated with PBS.

selective inhibitor of MEK, a kinase of the MAP kinase pathway from the IGF1R, did not affect IGF1-inhibited or basal expression of CTGF mRNA ($P > 0.1$). Addition of LY294002, a selective inhibitor of PI3K, a key player of the PI3K-Akt pathway downstream from the IGF1R, blocked IGF1 inhibition of CTGF mRNA expression ($P < 0.05$) (Fig. 1), indicating that the PI3-K-Akt pathway from the IGF1R mediates IGF1 inhibition of CTGF mRNA expression in MAC-T cells. LY294002 also increased ($P < 0.05$) CTGF mRNA expression in MAC-T cells in the absence of IGF1 (Fig. 1), suggesting that basal expression of CTGF mRNA in those cells is also inhibited by the PI3K signaling pathway. Rapamycin, an inhibitor of the mammalian target of rapamycin kinase (mTOR), had no effect ($P > 0.1$) on either IGF1-inhibited or basal expression of CTGF mRNA (Fig. 1), suggesting that a downstream component other than mTOR mediates PI3K-Akt inhibition of CTGF mRNA expression in MAC-T cells.

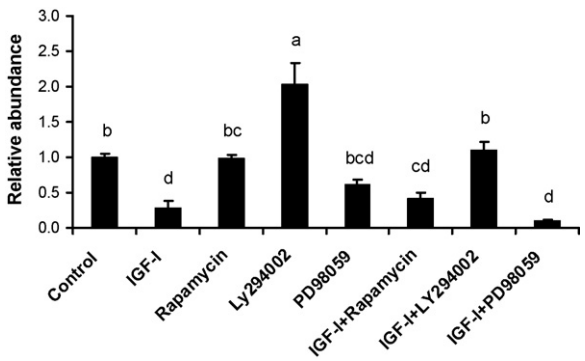

Fig. 1. Identification of the signaling pathway that mediates IGF1 inhibition of *CTGF* mRNA expression in MAC-T cells. The MAC-T cells were treated with IGF1, MEK inhibitor PD98059, PI3K inhibitor LY294002, or mTOR inhibitor rapamycin, alone or in combination for 16 h. The control received PBS and DMSO, the vehicles for IGF1 and the inhibitors, respectively. *CTGF* and *GAPDH* (internal control) mRNAs were quantified by quantitative real-time RT-PCR. Abundance of *CTGF* mRNA was normalized to that of *GAPDH* mRNA. Means ($n = 3$) labeled with different letters were significantly different ($P < 0.05$).

3.3. *IGF1 might also inhibit CTGF mRNA expression in bovine mammary tissue*

To determine whether IGF1 also inhibits CTGF mRNA expression in the mammary gland, we compared CTGF mRNA abundance in mammary parenchyma from cows injected with GH, which was expected to increase circulating IGF1 concentrations, with expression in control cows injected with excipient. As shown in Fig. 2A, the mammary tissue of GH-injected cows tended to express lower levels of CTGF mRNA than mammary tissue of control cows ($P = 0.07$). To exclude the possibility that GH directly inhibits CTGF mRNA expression in the mammary gland, we determined if GH affected CTGF mRNA expression in MAC-T cells that were rendered highly GH responsive by cotransfection with GH receptor and STAT5. As shown in Fig. 2B, GH had no effect on CTGF mRNA expression in the transfected MAC-T cells ($P > 0.1$). These data suggest that IGF1 might similarly decrease CTGF mRNA expression in the mammary gland in cows. Obviously, these data do not exclude the possibility that GH inhibits CTGF mRNA expression in the mammary gland through factors other than IGF-I.

3.4. *Effect of CTGF on IGF1-induced proliferation of MAC-T cells*

As indicated previously, CTGF might modulate the effect of IGF1 on MAC-T cells via its action as an IGF1-binding protein. To test this possibility, we determined the effects of IGF1 and CTGF, alone and in combination, on proliferation of MAC-T cells. As shown in

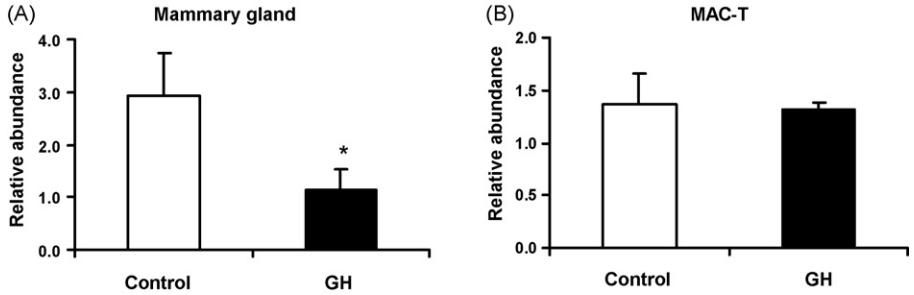

Fig. 2. Effects of GH on *CTGF* mRNA expression in bovine mammary tissue and MAC-T cells. (A) Effect of GH administration on mammary expression of *CTGF* mRNA in cows. Six lactating cows were injected intramuscularly with recombinant bovine GH or excipient (control) for 5 consecutive days before the mammary tissue was collected. Abundances of *CTGF* and *GAPDH* (internal control) mRNAs were measured by quantitative real-time RT-PCR. "*" indicates a tendency of significant difference ($P = 0.07$). (B) Effect of GH on *CTGF* mRNA expression in MAC-T cells. MAC-T cells were rendered highly responsive to GH by transfection with *GHR* and *STAT5* expression plasmids. The cells were treated with PBS (control) or GH for 16 h. *CTGF* and *GAPDH* (internal control) mRNAs were quantified by quantitative real-time RT-PCR. Abundance of *CTGF* mRNA was normalized to that of *GAPDH* mRNA. Mean *CTGF* mRNA abundance ($n = 4$) was not different between GH-treated and control MAC-T cells ($P > 0.1$).

Fig. 3, the cell cultures treated with 200 ng/mL of IGF1 or 200 ng/mL of CTGF had 45% or 20% more MAC-T cells, respectively, than cultures treated with PBS ($P < 0.05$). The cultures treated with 200 ng/mL of IGF1 and 200 ng/mL of CTGF together had 28% more viable cells than the PBS-treated cultures (Fig. 3). This latter percentage increase is smaller ($P < 0.05$) than the percentage increase (45%) by 200 ng/mL of IGF1 but greater ($P < 0.05$) than the percentage increase (20%) by 200 ng/mL of CTGF (Fig. 3). These results indicate that IGF1 and CTGF each stimulate proliferation of MAC-T cells, but in combination CTGF may antagonize the effects of IGF1 on cell proliferation.

Because CTGF is a binding protein of IGF1, a potential mechanism by which CTGF antagonizes effects of IGF1 on cells is through reducing the availability of IGF1 to IGF1R. If this is the case, excess IGF1 should overcome the competitive antagonism of CTGF on IGF1 stimulation of proliferation of MAC-T cells. As shown in Fig. 4, the cultures treated with 300 ng/mL of IGF1 and 200 ng/mL of CTGF contained more cells than the cultures treated with 200 ng/mL of IGF1 and 200 ng/mL of CTGF ($P < 0.05$), and contained similar numbers of cells as the cultures treated with 200 ng/mL of IGF1. As the concentration of IGF1 was further increased, the number of viable MAC-T cells also increased (Fig. 4). These data demonstrate that excess IGF1 can surmount the apparently antagonizing effect of CTGF on IGF1 stimulation of proliferation of MAC-T cells.

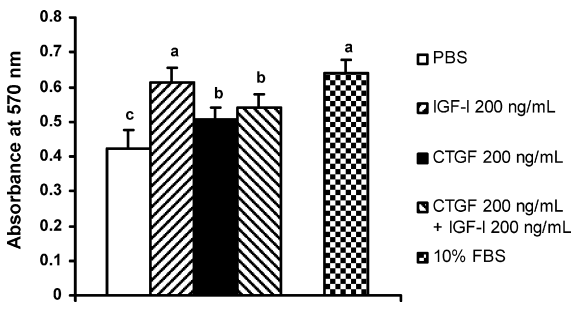

Fig. 3. Effect of CTGF on IGF1 stimulation of MAC-T cell proliferation. The cells were treated with PBS (control), 200 ng/mL of IGF1, 200 ng/mL of CTGF, 200 ng/mL of IGF1 plus 200 ng/mL of CTGF, or 10% FBS (positive control) for 16 h. Viable cells were quantified by a nonradioactive cell proliferation assay. Absorbance at 570 nm on the *y*-axis corresponds linearly to the number of viable cells. Means ($n = 4$) labeled with different letters are significantly different ($P < 0.05$).

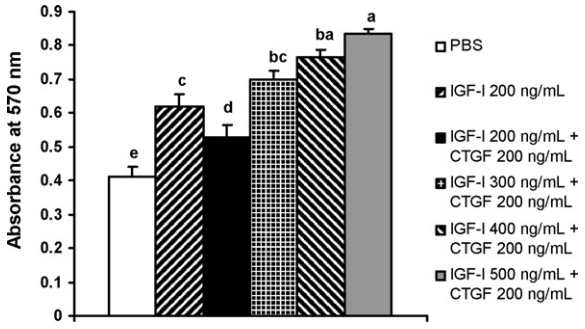

Fig. 4. Excess IGF1 overcame the antagonistic effect of CTGF on IGF1 stimulation of proliferation of MAC-T cells. The cells were treated with PBS (control), 200 ng/mL of IGF1, or 200 ng/mL of CTGF plus increasing concentrations of IGF1 for 16 h. Viable cells were quantified by a nonradioactive cell proliferation assay. Absorbance at 570 nm on the *y*-axis corresponds linearly to the number of viable cells. Means ($n = 4$) labeled with different letters are significantly different ($P < 0.05$).

3.5. Effects of CTGF on IGF1 activation of IGF1R

To determine the possibility that CTGF attenuates the effects of IGF1 on MAC-T cells by reducing its availability or its ability to activate the IGF1R, we quantified the abundance of phosphorylated IGF1R in MAC-T cells that were treated with IGF1 and CTGF, alone or in combination. As shown in Fig. 5, phosphorylated IGF1R was readily detectable in IGF1-treated MAC-T cells, but was barely detectable in CTGF- or PBS-treated MAC-T cells, indicating that unlike IGF1, CTGF does not activate IGF1 receptor. The assay also showed that CTGF had no effect on the levels of IGF1-activated phospho-IGF1R (Fig. 5), suggesting that CTGF does not reduce the availability of IGF1 to the IGF1R or the ability of IGF1 to activate IGF1R. Furthermore, CTGF did not affect the levels of total IGF1R protein in MAC-T cells (Fig. 5), excluding the possibility that CTGF inhibits IGF1 action by reducing IGF1R expression. The same analyses also showed that IGF1 did not affect IGF1R expression in MAC-T cells (Fig. 5).

4. Discussion

Originally isolated as a secretory product of endothelial cells that acts as a growth factor on fibroblasts [7], CTGF is now known to be produced by and to act on many cell and tissue types [15]. In this study, we have shown that CTGF mRNA is expressed in the bovine mammary epithelial cell line, MAC-T cells, and in the bovine mammary epithelia, and that its expression in MAC-T cells and probably also in the mammary tissue is inhibited by IGF1, a well-established regulator of mammary development and function. These results suggest that CTGF may play a role in development and physiology of the bovine mammary gland.

CTGF has both proliferative and apoptotic effects, depending on the type of target cell [15]. CTGF stimulates proliferation, migration, and adhesion of fibroblasts, chondrocytes, and endothelial cells [15], but induces apoptosis of vascular smooth muscle cells [25,26] and the human breast cancer cell line MCF-7 cells [27]. In this study, CTGF stimulated proliferation of MAC-T cells in the absence of any other growth factors, suggesting that CTGF per se is a proliferative factor for MAC-T cells. This proliferative effect of CTGF on the bovine mammary epithelial cells seems to differ from the effect of CTGF on murine or human mammary epithelial cells. Wang et al. [28] reported that CTGF induces lactogenic differentiation of the mouse mammary epithelial cell line, HC11 cells [28] and Hishikawa et al. [27] reported that overexpression of CTGF in MCF-7 cells

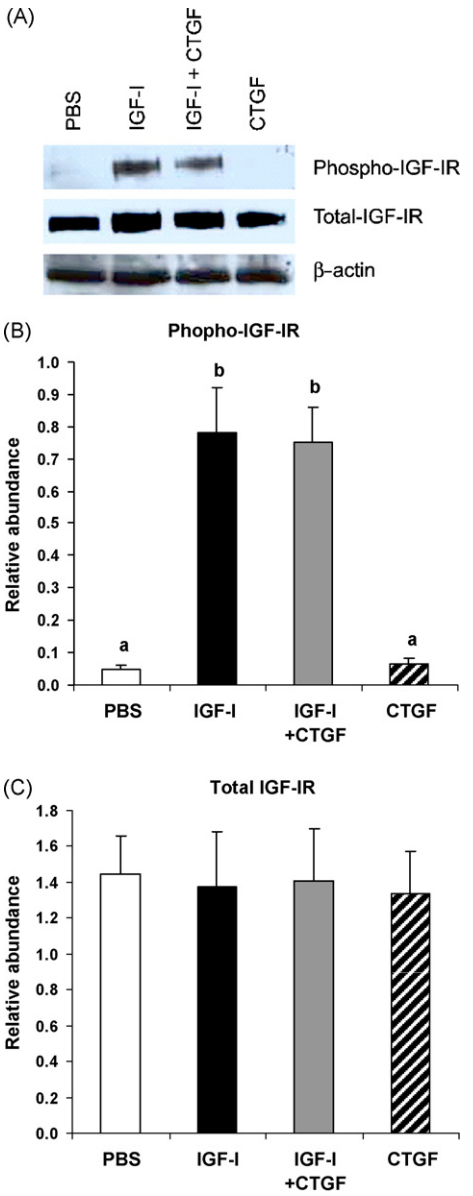

Fig. 5. Effect of CTGF on IGF1 activation of IGF1 receptor (IGF1R) expression in MAC-T cells. The cells were treated with PBS, 200 ng/mL of IGF1, 200 ng/mL of CTGF, or 200 ng/mL of IGF1 plus 200 ng/mL of CTGF for 1 h before being lysed for total cellular protein extraction. Phospho-IGF1R, total IGF1R and β-actin (loading control) protein abundances were detected by Western blotting analysis using specific antibodies. (A) Representative images of Western blotting analyses. The phospho-IGF1R, total IGF1R, and β-actin were detected from the same membrane. (B) Densitometric analysis of phospho-IGF1R protein bands from three experiments. In this analysis, the abundance of phospho-IGF1R protein was normalized to that of total IGF1R protein in the same sample. Means ($n = 3$) labeled with different letters are significantly different ($P < 0.05$). (C) Densitometric analysis of total IGF1R protein bands from three experiments. In this analysis, abundance of total IGF1R protein was normalized to that of β-actin. There is no significant difference between the mean values ($P > 0.1$).

induces apoptosis. These reports suggest that CTGF is a differentiation or apoptotic factor for murine mammary epithelial cells or human breast cancer epithelial cells, respectively. These differences may reflect species-specific effects of CTGF on the same tissue or cell type, differences between normal and cancer cells, or simply indicate that the effects of CTGF on mammary cell lines do not recapitulate the functions of CTGF in the mammary gland.

CTGF expression is up-regulated by transforming growth factor-β1 (TGFB1) in several fibroblast cell lines [29], primary fibroblasts [30,31], and fibrotic disorders involving inflammation and connective tissue accumulation [32]. TGFB1-stimulated collagen production in fibroblasts is blocked by anti-CTGF antibodies or antisense oligonucleotides [33]. Therefore, CTGF is widely considered to be a downstream mediator of the actions of TGFB1, especially in the promotion of fibroblast proliferation and extracellular matrix production [34]. In this study, we found that CTGF mRNA expression in MAC-T cells is robustly inhibited by IGF1 and that this inhibition is mediated specifically through the PI3K-Akt signaling pathway from the IGF1R. These observations suggest that CTGF and IGF1 may be functionally related in the bovine mammary gland. We also showed that although CTGF stimulates MAC-T cell proliferation by itself, it attenuates the stimulatory effect of IGF1 on proliferation of MAC-T cells. Therefore, a functional relationship between CTGF and IGF1 in the bovine mammary gland may be that, by inhibiting CTGF expression, IGF1 reduces the antagonistic effect of CTGF on IGF1 stimulation of mammary epithelial cell proliferation.

One major effect of IGF binding proteins is to modulate the availability of IGF1 to its receptor [5,6]. In this study we observed that, although CTGF attenuates the effect of IGF1 on cell proliferation, this attenuation can be reversed by excess IGF1. These observations suggest that CTGF, produced by MAC-T cells, functions as a binding protein to inhibit the availability to IGF1 to its receptor on MAC-T cells, thereby attenuating IGF1 stimulated proliferation of MAC-T cells. However, we also found that CTGF has no effect on IGF1-induced phosphorylation of IGF1R protein, arguing against an inhibitory effect of CTGF on the availability of IGF1 to its receptor or the activity of IGF1 on it membrane receptor. We have also shown that CTGF does not affect IGF1R expression in MAC-T cells. Taken together, these observations suggest that a post-IGF1R mechanism mediates the attenuating effect of CTGF on the stimulation of MAC-T cell proliferation by IGF1. Interestingly, CTGF has been reported to suppress IGF1-induced phosphorylation of Akt and ERK in non-small cell lung cancer (NSCLC) cell lines [35]. It remains to be determined if CTGF has the same effect on IGF1-induced phosphorylation of Akt and ERK in MAC-T cells. Because CTGF by itself stimulates MAC-T cell proliferation, another potential mechanism by which it may attenuate the stimulatory effect of IGF1 on MAC-T cell proliferation is through an overlap between signaling pathways used by the two growth factors to stimulate MAC-T cell proliferation; whereby when both factors are present one competitively inhibits the effect of the other.

In summary, the results of this study suggest a novel biochemical and functional relationship between CTGF and IGF1 in the bovine mammary gland. We have shown that IGF1 inhibits CTGF mRNA expression in the bovine mammary epithelial cell-derived cell line MAC-T cells and probably also in the bovine mammary gland, and that this effect is mediated through the PI-3K-Akt signaling pathway. We have also shown that although CTGF is a proliferative factor by itself, it attenuates IGF1's ability to stimulate MAC-T cell proliferation. This effect is not mediated through reduced binding of IGF1 to IGF1R or reduced IGF1R expression. Inhibition of CTGF expression may be part of a mechanism by which IGF1 stimulates proliferation of epithelial cells in the mammary gland.

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